遗 传 学 报 *Acta Genetica Sinica*, December 2006, 33 (12): 1081–1086 ISSN 0379-4172

Analysis of the Genetic Diversity and the Phylogenetic Evolution of Chinese Sheep Based on Cyt *b* Gene Sequences

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Abstract: The complete sequences of Cyt *b* gene from 20 individuals belonging to eight Chinese indigenous sheep breeds and one foreign breed were studied. The results showed that the hapolotype diversity of Chinese sheep breeds was 97.1%. The mean nucleotide composition of all the sequences was 27.1% T, 28.5% C, 31.4% A, and 13.0% G. The nucleotide diversity was 0.602%. A total of 43 mutation sites were detected, including 40 transitions and 3 transversions. Fu's test of selective neutrality showed that the sheep populations had no population demographic expansion ($0.10 > P > 0.05$). The different clustering methods, namely neighbor-joining, minimum evolution, and unweighted pair group method with arithmetic means, all showed a similar result, which indicated that Chinese local sheep had three maternal resources.

Key words: sheep; Cyt *b*; phylogenetic evolution

Mitochondrial DNA (mtDNA) is the genetic material that exists outside the nucleus in eukaryotic cells. It has a simple molecular structure. It does not undergo recombination with nuclear DNA and has no identical sequence with nuclear DNA. It has multiple copies, has a rapid evolutionary rate, and follows maternal inheritance. Cytochrome *b* gene (Cyt *b*) is one of the genes that is coded by mtDNA, and its gene product plays an important role in electron transfer in the respiration chain. Cyt *b* gene has a moderate evolutionary rate and a clear evolutionary pattern that makes it suitable for the studies on the phylogenetic evolution at the intra- and interspecific levels[1-3].

China has a centuries-old history of breeding sheep. It has abundant sheep breed resources, with more than 40 local sheep breeds, which serve as important genetic resources for the sustainable development of animal husbandry and for the preservation of biological diversities[4]. For example, Ganjia and Oula sheep in Gansu Province belonging to local breeds of Tibetan sheep can adapt to the plateau environment and endure coarse feeding. The Bashibai sheep, which is found in Xijiang Province, has delicate and tasty meat that is easier to digest and has a higher calcium and iron content. In addition, there are other local sheep breeds, such as Henan big-tail sheep and Heiqiupi sheep that possess unique features. Previous studies on these breeds were carried out only at the morphological level. There are still no reports on some of the breeds at the molecular level. The purpose of this study was to investigate the genetic diversity and phylogenetic evolution of Chinese sheep based on the analysis of the complete sequence of the Cyt *b* gene. This will be helpful for the conservation, utilization, and exploitation of the genetic resources of the indigenous Chinese sheep.

Received: 2005-11-23; Accepted: 2006-05-20

This work was supported by the National Natural Science Foundation of China (No. 30371026), Doctor Startup Fund (No. 01140503) and Topnotch Person with Personal Ability Support Project of Northwest A&F University (No. 01140101).

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1 Materials and Methods

1.1 Sample collection and DNA extraction

According to the simple random sampling method, ear samples from 382 individuals of nine different sheep breeds were collected at livestock farms throughout China. The breeds were chosen from Xinjiang Province (Bashibai sheep, *n*=48; Tashikuergan sheep, *n*=47), Gansu Province (Lanzhou big tail sheep, *n*=42; Ganjia sheep, *n*=47; Oula sheep, *n*=48; and Heiqiupi sheep, *n*=48), Shaanxi Province (Hanzhong sheep, *n*=50), Henan Province (Henan big tail sheep, *n*=52), and Beijing City (skudde, *n*=30). DNA was extracted from these specimens using phenol–chloroform method[5].

1.2 Amplification, purification, cloning, and DNA sequencing

Primers for Cyt *b* gene were designed using Primer 5.0 software and synthesized by Shanghai Sangon Bio-tech Co., Ltd. The length of the amplified fragment was about 1 610 bp, including the complete Cyt *b* gene (1 140 bp) and more than 230 bp sequence in both flanks. The sequences of the primers were forward: 5′-ACACCCAACCCCACCAC-3′, reverse: 5′-GTGGGTGGTTGTGCTTTTCT-3′. The volume of the PCR amplification reaction system was 60 μL, consisting of genomic DNA 50 ng, dNTPs 200 μmol/L, primers 10 pmol, $MgCl_2$ 250 μmol/L, and *Taq* DNA polymerase 1 U. The reaction conditions included an initial denaturation at 95℃ for 5 min, followed by 30 cycles, each consisting of 30 s denaturation at 95℃, primer annealing at 56℃ for 45 s, extension at 72℃ for 60 s, and then a final extension at 72℃ for 8 min. The PCR products were electrophoresed using 2.0% (wt/vol) agarose gel, which was stained with ethidium bromide solution.

The amplified products were purified with a DNA purification kit according to the manufacturer's instructions (TW-Biotech Co., Ltd.). The purified fragments were cloned into pGEM-T easy vector and subsequently transformed into *E. coli* Top10. After 16–20 hours, single colonies were inoculated to obtain recombinant plasmid. The recombinant DNA was extracted and sequenced using an ABI model 3730 automated sequencer. The results that were indicative of unique polymorphisms were confirmed by resequencing of the independent clones.

1.3 Data analysis

The sequences were aligned using the BioEdit 7.0 software. Identical sequences were considered as belonging to the same haplotype. The DnaSP 4.1 program was used to analyze the polymorphism of the haplotypes and to estimate the degree of variation and the substitution frequency of the nucleotides. The molecular phylogenetic trees were constructed to analyze evolution of sheep using the MEGA 2.0 program with a Kimura 2-parameter model[6]. The bootstrap value was 1 000.

2 Results

2.1 Nucleotide analysis of Cyt *b* gene

When compared with the control sequence (Accession No. NC001941), a total of 43 polymorphic sites were obtained from the 21 sequences（from top to bottom in Fig. 1, the GenBank accession numbers range from DQ903208 to DQ903227 except NC001941), including 29 single variable sites and 14 parsimony informative sites (Fig. 1). Transversions occurred only at three positions: 603 (C/G), 693 (T/G), and 1078 (C/A) and in all the other positions, transitions occurred (G/A, 16 and T/C, 24). The transitions of T/C and A/G at positions 309 and 495 could be regarded as characteristics of haplotype B, and transitions of T/C at positions 207, 393, 396, and 696; C/T at 328, 735, and 990; A/G at 476 and 813; and G/A at 939 could be regarded as characteristic of haplotype C.

The variance of nucleotide diversity was 3.77%, the transition rate was 3.51% and the transversion rate was 0.26%. There were 24 synonymous and 19 nonsynonymous substitutions among the 43 variations, and the nucleotide diversity of synonymous and